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37 C.F.R. §1.8

I hereby certify that this correspondence is being sent via facsimile to Examiner Hallry J. Guttman, Ph.D. at (703) 308-4242 with the United States Patent and Trademark Office on the date below:

December 20, 2001
Date

Steven L. Highlander

**PATENT**

# IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

*In re* Application of:
Theodore W. RANDOLPH *et al.*

Serial No.: 09/350,327

Filed: July 9, 1999

For: HIGH PRESSURE REFOLDING OF PROTEIN AGGREGATES AND INCLUSION BODIES

Group Art Unit: 1651

Examiner: H. Guttman

Atty. Dkt. No.: UTEC:003/SLH

Considered

10-Jan-02

## SECOND DECLARATION OF THEODORE W. RANDOLPH

Hon. Commissioner for Patents
Washington, D.C. 20231

I, Theodore W. Randolph:

1. I am the Theodore W. Randolph listed as an inventor on the above-captioned application. I currently hold the position of Professor in the Department of Chemical Engineering at the University of Colorado (Boulder).

2. I understand that the examiner in charge of the above-captioned application continues to question the enablement of the present claims. In particular, I understand that the examiner has raised questions regarding the enablement of two-step pressurization methods, and the

broad application of the claimed methods to proteins in general. In response, I would like to provide the following information for the examiner's consideration.

**Lysozyme**

3. In the original application, Example 3 describes the depressurization protocol used in refolding experiments. We now provide additional details as to how the previously described 10 bar/minute depressurization rate was achieved in the specific case of lysozyme refolding from covalently-crosslinked aggregates.

4. Pressure was generated using high-pressure nitrogen (40 MPa) connected to a 10-fold hydraulic intensifier (High Pressure Equipment Company, Erie, PA). One mg/ml suspensions of lysozyme aggregates in 50 mM Tris (pH 8.0, at 24°C), varying amounts of GdnHCl, 2mM DTT and GSSG at the desired final GSSG:GSH ratio (an optimal ratio was 1:1) were prepared in heat-sealed bulbs of SAMCO® transfer pipets, sealed within a bag of water, and placed into a to a 2L cloverleaf reactor rated to 200 MPa and filled with oil. Samples were slowly pressurized (over 20 minutes) to final desired pressure to minimize pressurization-induced heating. Depressurization was conducted in 20 MPa increments, with each depressurization step requiring approximately 5 minutes. Samples were incubated at each intermediate pressure during depressurization for 15 minutes, yielding an overall depressurization rate of 1 MPa/minute (10 bar/minute). Thermal transients caused by pressure-induced heating were minimal (<2°C) at this pressurization rate, as monitored by thermocouples mounted in the pressure vessel. Unless otherwise stated, all pressure experiments were performed at 24°C.

5. The foregoing explanation is believed to demonstrate that the specification as filed did indeed provide evidence of a two-step method, in contrast to the examiner's assertions that it did not. The following examples are provided as further evidence of the enablement of this methodology.

**aVEGF**

6. High pressure refolding studies were conducted on non-native dimeric aggregates of an antibody against human vascular endothelial growth factor (aVEGF), a full-length, disulfide-bonded, glycosylated, heterotetrameric antibody that undergoes irreversible dimerization during production in chinese hamster ovary (CHO) cell culture. Refolding studies were conducted to determine if high hydrostatic pressure could be used to obtain native aVEGF monomers. Additionally, experiments were run to determine the effect of temperature on refolding yield. Studies were conducted by Mr. Matthew Seefeldt, a first-year graduate student with a background in petrochemical engineering, using the patent application U.S. Serial No. 60/092,208, filed July 9, 1998, for guidance in choosing ranges of experimental parameters.

7. Experiments were conducted to determine the refolding yield of samples held at 2000 bar at three different temperatures. The experimental procedure was as follows. aVEGF (22% aggregate, 78% monomer) was diluted to a concentration of 1mg/ml in 25mM 2-(4-Morpholino)-Ethane Sulfonic Acid (MES) buffer at pH 6.0. Each sample was made of 0.4 ml of the MES protein solution (1 mg/ml) loaded into a sealed syringe. The syringe is required to ensure proper pressure transfer to the sample. Three samples were created for each condition and placed into pressure vessels at room temperature. The samples

were pressurized to 2000 bar and the pressure vessel temperature adjusted to 0°C, 25°C, or 50°C. The pressure was monitored during this time to maintain 2000 bar despite temperature changes. The samples were held at the desired temperature and pressure for sixteen hours, cooled or warmed to room temperature, and depressurized. Depressurization was done by 100 bar increments every thirty minutes, with drops of 250 bar every fifteen minutes once 1000 bar was reached. The samples were removed from the pressure vessels and stored at 4°C for two days. Size exclusion chromatography (SEC) was used to determine the mass fractions of monomers and dimers.

8. Three samples were tested at 0°C, 25°C, and 50°C and 2000 bar through the procedure described above. Refolding yield (RY%) was calculated from the SEC results using the following equation:

$$RY\% = \frac{M_f - M_i}{1 - M_i} * 100$$

where $M_f$ = final monomer fraction, and $M_i$ = initial monomer fraction

Temperature was found to influence the refolding yield. These studies showed that one can achieve a refolding yield of 29% (+/- 1%) at 50°C. This is shown in FIG. 1 below. Thus, high hydrostatic pressure can reverse antibody dimers, obtaining a refolding yield of 29% (+/- 1%). This study verified the impact of both elevated and low temperatures on refolding yield and provides a case study of the effect of pressure on an antibody system.

**Bikunin**

9. Refolding studies have been conducted on bikunin, a 170 amino acid protein with six disulfide bonds. This protein often forms an aggregate during fermentation. The aggregate is composed of non-native disulfide bonds and is an oligomer of four to eight monomers. Experiments were conducted to determine if high hydrostatic pressure (1000-3000 bar) could be used to refold these disulfide scrambled protein aggregates.

10. Aggregated bikunin was placed in sealed syringes and held under pressure at various reactor conditions. Pressure, pH, redox conditions, temperature and depressurization rate were all controlled. As a base case, the following conditions were used: 1 mg/ml sample of aggregated bikunin was held at 200 MPa, 25°C, in 4 mM oxidized glutathione (GSSG), 2 mM dithiothreitol (DTT) for 16 hours. The samples were then depressurized by 10 MPa every 30 minutes until a pressure of 100 MPa was reached. The samples were further depressurized 25 MPa every fifteen minutes until 0.1 MPa was reached. After pressurization, the samples were analyzed with size exclusion chromatography (SEC) to determine the refolding yield. A calibration curve was used to ensure that mass balance was maintained during experimentation. Samples were also sent to a secondary lab for analysis through reverse phase chromatography (RP) and activity assay. These secondary studies showed that SEC over-estimated the refolding yield by about 10% due to the presence of non-active monomeric proteins. SEC was still used as an optimization tool; however, final refolding yields were confirmed with an activity assay. A refolding yield

of 45% +/- 4% was obtained as measured by activity assay at the "base case" conditions described above.

11. A rapid optimization of refolding conditions was obtained by varying solution conditions around the "base case" conditions set out above. An unsophisticated algorithm was used; more sophisticated designed experiments well-known to industrial scientists would likely yield more optimal conditions with fewer experiments. However, even using the simple technique of varying redox conditions, temperature, pressure and depressurization rate separately around the base case conditions, a reasonable optimum was quickly found.

12. Oxidized and reduced glutathione can be used to control the redox conditions within the refolding solution. These compounds are needed to break non-native disulfide bonds and reform native disulfides. Typically, optimum redox conditions exist when the total glutathione concentration is between 6-16 mM, with ratios of reduced to oxidized glutathione specific to the system, often between 1 and 3. Samples were made with varying redox conditions and tested at three different pressure conditions to determine the effect on refolding yield. The ratio of the monomeric SEC peak to the aggregated SEC peak was used to measure the effectiveness of refolding. The results are shown in Figure 2. For these studies, the most optimum case was found to be 4 mM oxidized glutathione, 2 mM DTT at 2000 bar.

13. The glutathione shuffling system was necessary to obtain any significant refolding yield, as would be expected for a protein aggregate containing non-native disulfide bonds. Pressure and DTT alone were not effective. Further optimization of the redox conditions

could potentially be done; however the yields obtained in 4 mM GSSG, 2 mM DTT at 2000 bar were adequate to determine the effects of the remaining refolding conditions.

14. The effect of refolding temperature was evaluated. Samples were held at 12 hours at varying refolding temperatures, then brought to room temperature and held for an additional twelve hours. Pressures of 2000 bar and the previously optimized redox conditions were used. Refolding was found to be maximal at 25°C. These results are shown in Figure 3.

15. The effect of refolding pressure was evaluated. Samples were held at 16 hours at varying refolding pressures, and then slowly depressurized over an eight-hour period. The samples were held at room temperature, with the redox conditions previously used. A pressure of 2000 bar was found to be the optimum, with refolding yield decreasing at both 1000 bar and 3000 bar. These results are shown in Figure 4.

16. Protein monomer was subjected to rapid and slow depressurization rates to determine the impact of depressurization of protein yield. Samples were held at 2000 bar, 25°C, for 16 hours in the standard redox conditions (4 mM GSSG, 2 mM DTT). One set of samples was depressurized over a thirty second period. The second set of samples was depressurized by 10 MPa every 30 minutes until a pressure of 100 MPa was reached, then further depressurized 25 MPa every fifteen minutes until 0.1 MPa was reached. The samples that were depressurized slowly lost 21% +/- 3% of the monomer to aggregates. This value was much lower than the 59% loss +/- 16% when the sample was

depressurized quickly. This study verified the need to use slow depressurization over a period of hours to ensure that the proteins maintain their native conformation.

**GCSF**

17. Concentrated, purified inclusion bodies of granulocyte colony stimulating factor (GCSF) supplied from Amgen, Inc. (Thousand Oaks, CA) were suspended in refolding buffer (50 mM Tris-HCl, 1 mM EDTA, 0.1% $NaN_3$) containing varying concentrations of guanidine hydrochloride (GdnHCl). Triplicate samples were pressurized to 2000 bar and incubated for 24 hours. Pressure was released by decreasing the vessel pressure in 200 bar increments. Samples were held at each incremental pressure for 15 minutes.

18. Upon complete depressurization, samples were centrifuged at 13,000g for 15 minutes. The resulting supernatant was analyzed with the Pierce® TPA total protein assay. Bovine serum albumin (BSA) standards were used to calibrate the assay. The results are show in Figure 5.

**Summary**

19. In conclusion, I again submit that the foregoing data provide evidence on two major issues raised by the examiner. First, the data show that a two-step depressurization method does, in fact, provide a working refolding process. Second, the ability to refold dimeric, heterotetrameric, glycosylated, and disulfide-bonded proteins demonstrates that even complex proteins are amenable to refolding using methods according to the present invention.

20. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

12-19-2001
Date

Dr. Theodore W. Randolph

## LEGENDS TO FIGURES

Figure 1 – Effect of temperature on aVEGF refolding. A yield of 29% was achieved for samples held at 2000 bar and 50°C. Error bars denote 95% confidence interval.

Figure 2 – Effect of redox conditions at various pressures on refolding yield. Maximum yield was obtained for samples in 4 mM GGSG, 2 mM DTT and held at 2000 bar.

GSSG

Figure 3 – The effect of temperature on refolding yield. Maximum yield was obtained when the refolding temperature was held at 25°C.

Figure 4 – Effect of pressure on refolding yield. Refolding yield was found to be a maximum at 2000 bar.

Figure 5 – Solubilized Protein from GCSF inclusion bodies. Circles (●) represent atmospheric samples; squares (■) represent samples incubated at 2000 bar for 24 hours.

**Effect of Temperature on the Refolding Yield of aVEGF Dimers (2000 Bar for 16h, Slow Depressurization)**

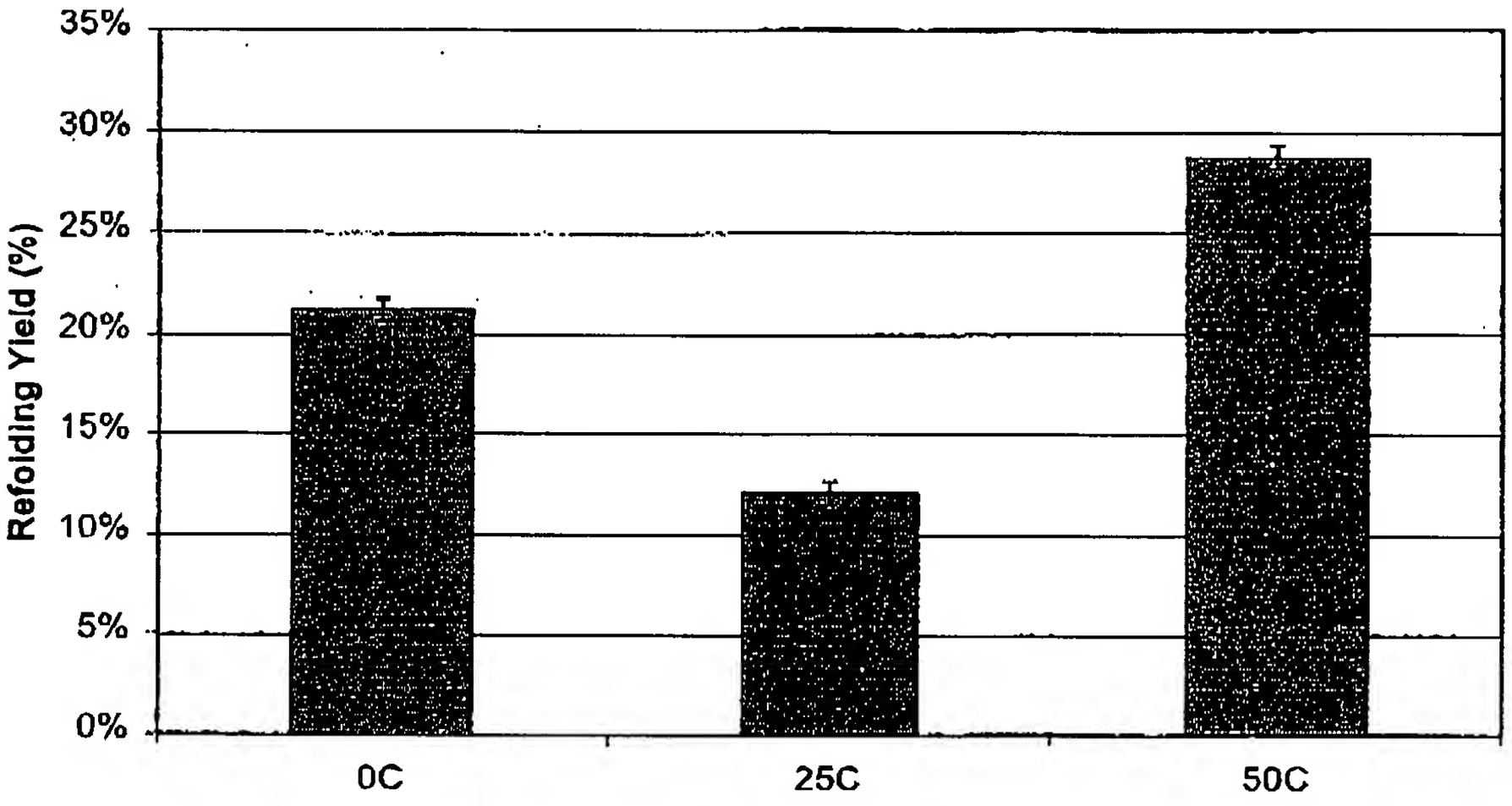


**Figure 1 – Effect of temperature on aVEGF refolding. A yield of 29% was achieved for samples held at 2000 bar and 50C. Error bars denote 95% confidence interval.**

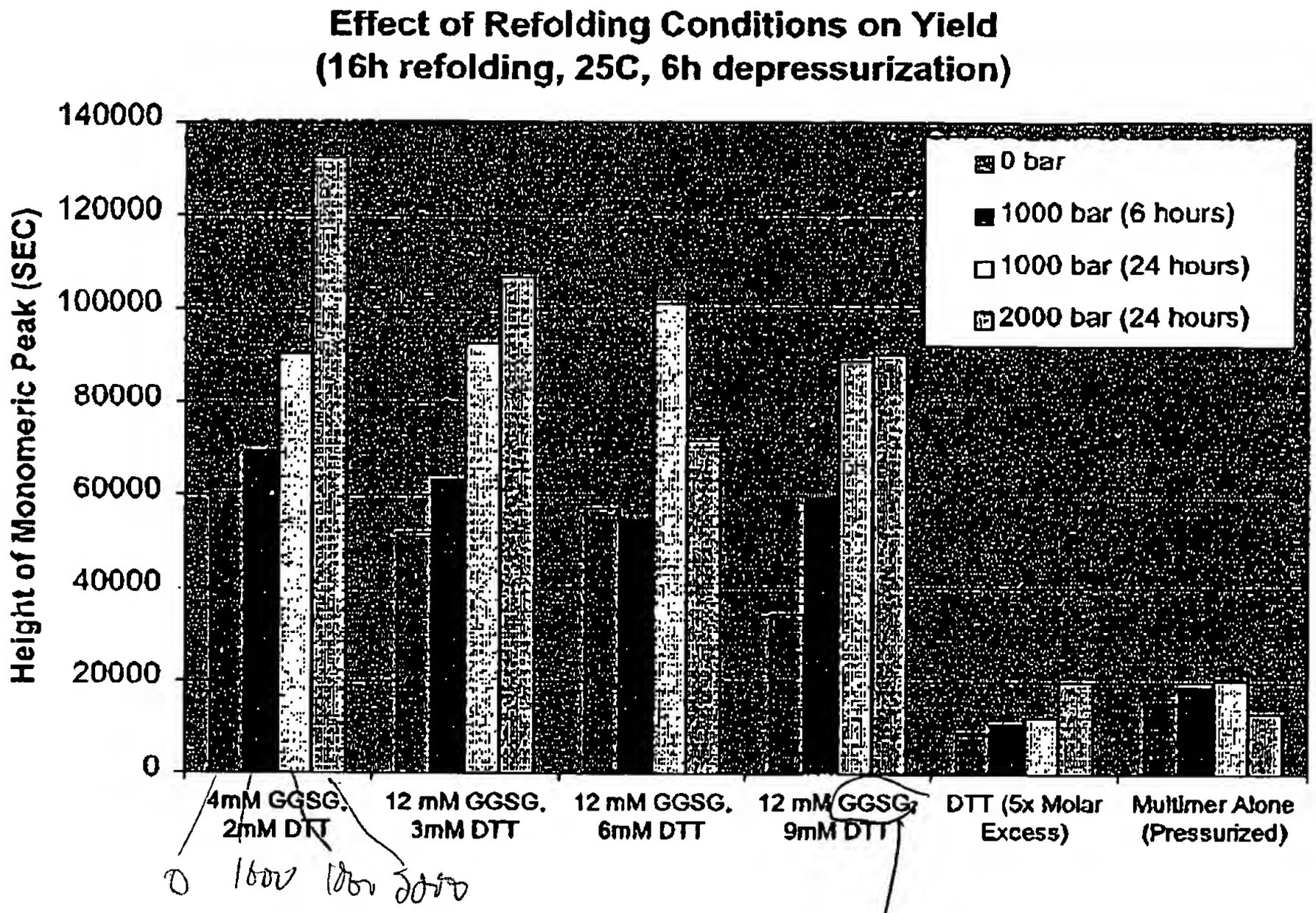


**Figure 2 – Effect of redox conditions at various pressures on refolding yield. Maximum yield was obtained for samples in 4mM GGSG, 2mM DTT and held at 2000 bar.**

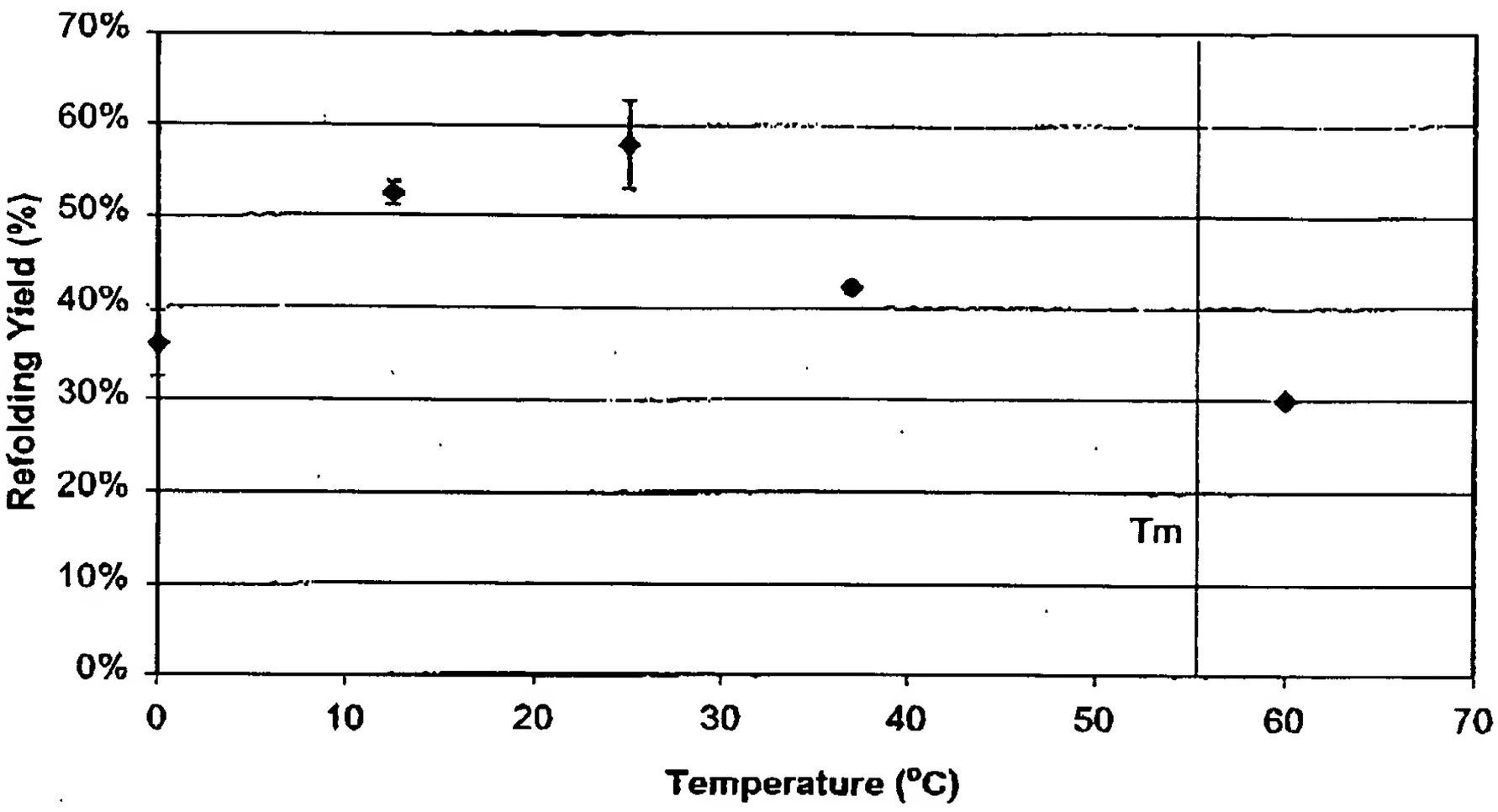


**Figure 3 – The effect of temperature on refolding yield. Maximum yield was obtained when the refolding temperature was held at 25°C.**

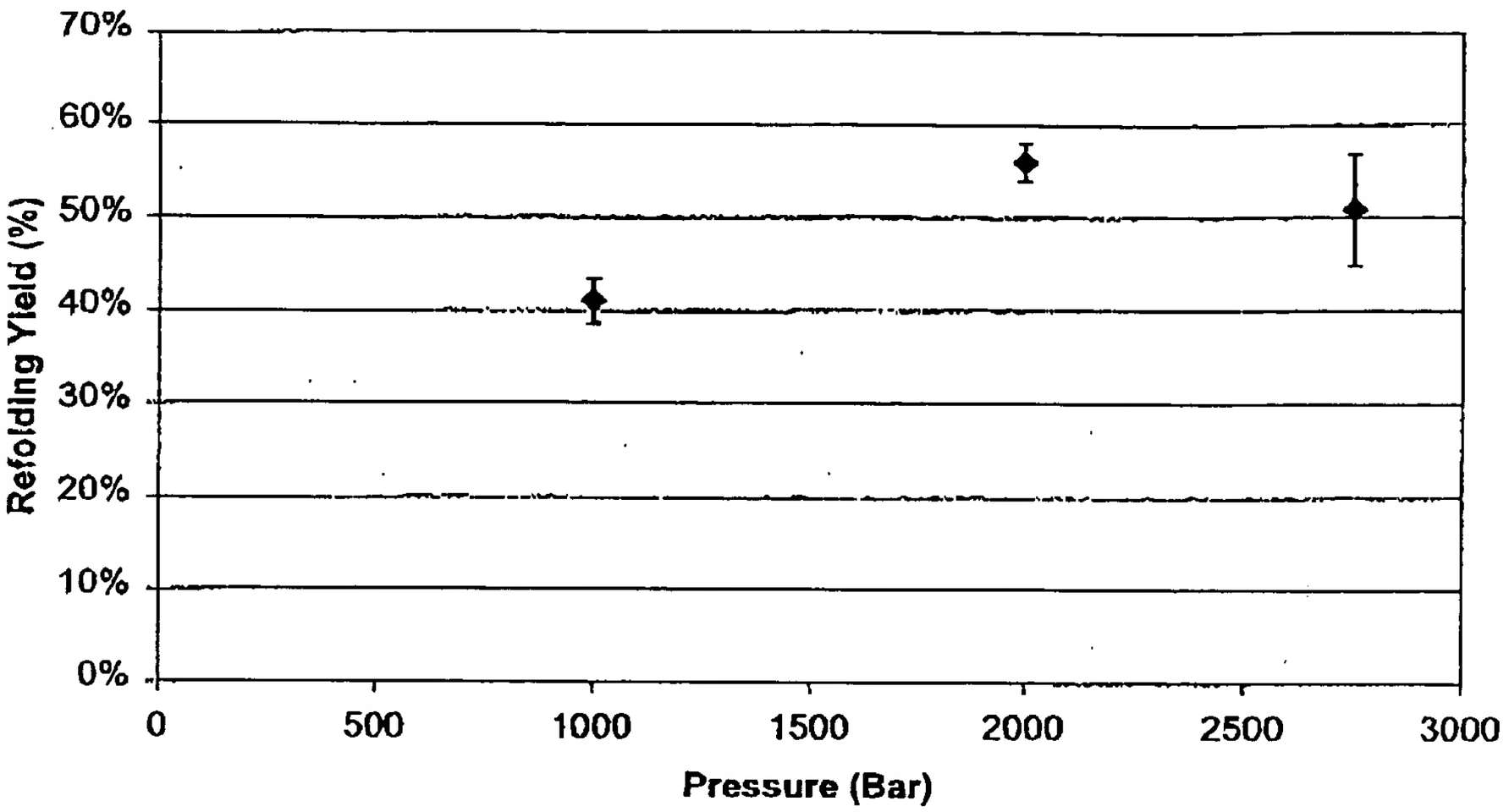


**Figure 4 – Effect of pressure on refolding yield. Refolding yield was found to be a maximum at 2000 bar.**

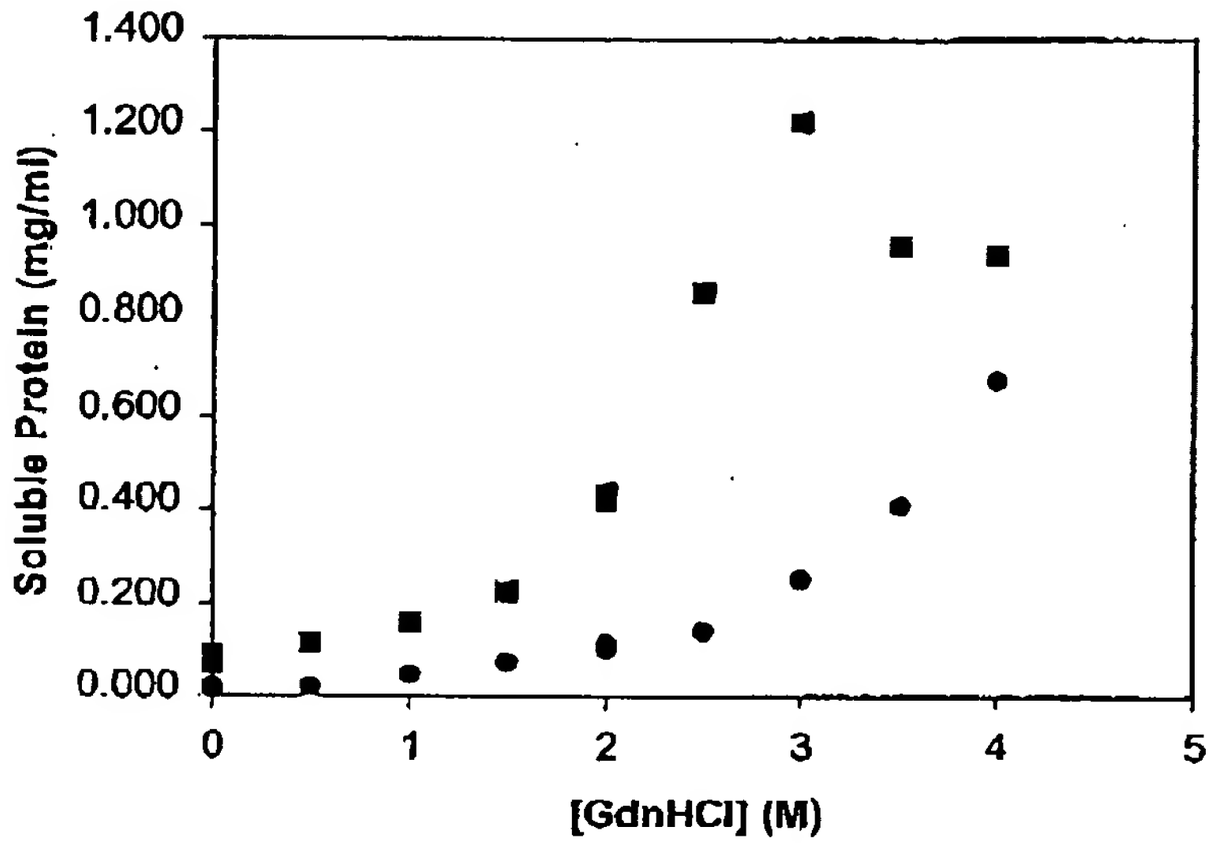


**Figure 5 - Solubilized Protein from GCSF inclusion bodies: Circles (•) represent atmospheric samples. Squares (■) represent samples incubated at 2000 bar for 24 hours.**